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(54) Title: TRANSGENIC ZEBRA FISH EMBRYO MODEL FOR HEMATOPOIESIS AND LYMPHOPROLIFERATIVE DISORDERS

(57) Abstract: A transgenic zebrafish animal model for the study of haemopoietic cell differentiation, control, and screening of therapeutic agents.



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# TRANSGENIC ZEBRA FISH EMBRYO MODEL FOR HEMATOPOIESIS AND LYMPHOPROLIFERATIVE DISORDERS

## Background of the Invention

Acute lymphoblastic leukemia (ALL) is the most common form of  
5 cancer in children (Pizzo and Poplack, 1993, Greaves, 1986, Uckun et al., 1998, Crist  
et al., 1988). A better understanding of the biological basis and predisposing  
leukemogenic events in this disease is needed in order to develop more effective  
treatment programs as well as novel prevention strategies.

Leukemic clones are thought to originate in ALL patients from  
10 normal lymphocyte precursors arrested at various stages of T- or B-lymphocyte  
development (Greaves, 1986). Accordingly, any critical regulatory network that  
controls normal lymphocyte development is a potential target for a leukemogenic  
event.

One such regulatory network vital for normal hematopoiesis involves  
15 Ikaros, a member of the Kruppel family "zinc finger" DNA-binding proteins. Ikaros  
acts as an evolutionarily conserved "master switch" of hematopoiesis that dictates  
the transcriptional regulation of lymphocyte ontogeny and differentiation  
(Georgopoulos et al., 1994, Georgopoulos et al., 1992, Hahm et al., 1994, Molnar  
and Georgopoulos, 1994, Wang et al., 1996, Winandy et al., 1995, Molnar et al.,  
20 1996, Sun et al., 1996, Hansen et al., 1997, Georgopoulos et al., 1997, Brown et al.,  
1997, Klug et al., 1998).

The programmed expression and function of the *Ikaros* gene is tightly  
controlled by alternative splicing of the Ikaros pre-mRNA which results in  
production of eight different Ikaros isoforms. All eight Ikaros isoforms share a  
25 common carboxy(C)-terminal domain containing a transcription activation motif and

two zinc finger motifs that are required for hetero- and homodimerization among the Ikaros isoforms and for interactions with other proteins (Hahn et al., 1994, Molnar and Georgopoulos, 1994, Sun et al., 1996). Only three of the eight Ikaros isoforms, however, contain the requisite three or more amino(N)-terminal zinc fingers that  
5 confer high affinity binding to an Ikaros-specific core DNA sequence motif in the promoters of target genes (Sun et al., 1996).

The formation of homo- and heterodimers among the DNA binding isoforms increases their affinity for DNA, whereas heterodimers between the DNA binding isoforms and non-DNA binding isoforms are unable to bind DNA.  
10 Therefore, Ikaros proteins with fewer than three N-terminal zinc fingers exert a dominant negative effect by interfering with the activity of Ikaros isoforms that can bind DNA (Molnar et al., 1996, Sun et al., 1996). Thus, splicing errors can have severe consequences for the lymphocyte compartment of the developing immune system. An abundance of dominant-negative Ikaros isoforms that no longer bind  
15 DNA could result in significantly impaired expression of regulatory target genes that are essential for the orderly development and maturation of lymphocyte precursors.

In mice, absence of the normal Ikaros gene results in an early and complete arrest in the development of all lymphoid lineages during both fetal and adult hematopoiesis (Georgopoulos et al., 1994). Ikaros-deficient mice have a  
20 rudimentary thymus, lack peripheral lymph nodes, and are characterized by a complete absence of lymphocyte progenitor cells as well as mature B-lymphocytes, T-lymphocytes, and natural killer cells (Georgopoulos et al., 1994). Mice heterozygous for a germline mutation which results in the loss of critical DNA-binding zinc fingers of Ikaros develop a very aggressive form of lymphoblastic

leukemia with a concomitant loss of the single wild type *Ikaros* allele between three and six months after birth (Winandy et al., 1995). Finally, the most recent findings in ALL molecular etiology show a pivotal role for *Ikaros* gene regulation in lymphoblast neoplastic transformation in infants (Sun et al., 1999) with T-lineage or  
5 B-lineage ALL leukemic cells expressing high levels of dominant-negative *Ikaros* isoforms.

It has long been suspected that molecular rearrangements in the lymphoid lineage precursors leading to ALL occur during fetal hematopoiesis (Ford et al., 1993, Gill Super et al., 1994). With the prospect of *Ikaros* malfunction and  
10 *Ikaros* isoform expression being at the core of leukemogenesis, a better understanding of the events taking place during embryonic blood cell differentiation is required in order to develop rational therapies. To address this need, an adequate experimental model system of vertebrate hematopoiesis is essential.

The zebrafish (ZF), with its extremely rapid embryonic development  
15 (3 days) and short maturation period (2-3 months) offers an attractive model. Over the past decade, the ZF embryo has been used to study eukaryotic gene activity and intercellular signaling in vertebrate development (Nusslein-Volhard, 1994, Zhang et al., 1998, Nguyen et al., 1998), and has emerged as a powerful genetic system, strongly relevant to the study of molecular medicine (Driever and Fishman, 1996,  
20 Amemiya, 1998). Intensive study of early embryonic hematopoiesis in the ZF along with the generation of hematopoietic mutants has turned the ZF into a useful model for the study of human blood disorders, such as congenital sideroblastic anemia (Brownlie et al., 1998) and hepatoerythropoietic porphyria (Wang et al., 1998). (See detailed reviews: Bahary and Zon, 1998, Amatruda and Zon, 1999).

It has now been discovered that transient, inappropriate expression during early embryonic development of the non-DNA binding Ikaros forms, including the dominant-negative isoforms, mutant forms, and others, have a significant impact on blood cell differentiation at later stages of development. Using the transgenic animal model of the invention, the effect of various agents on blood cell differentiation can be efficiently assessed. The ZF, with its relatively large and translucent embryo, external fertilization, and extracorporate development, provides a model of choice for transgenic research (Stuart et al., 1990, Culp et al., 1991, Hammerschmidt et al., 1999).

This model can be used, for example, to examine the impact of alteration of the Ikaros program of gene expression on definitive hematopoiesis in adults, within the short period of hematopoietic cell determination in ZF embryonic development. As described herein, a transgenic Zebrafish (ZF) animal model provides an excellent model of vertebrate hematopoiesis.

#### Summary Of The Invention

The present invention provides a useful animal model for the screening and study of hematopoiesis and agents capable of modulating hematopoietic development. In particular, the ZF embryo carrying an Ikaros transgene provides a model for the study and modulation of lymphocyte development and leukemia.

In one embodiment of the invention, the transgene is a DNA-binding Ikaros isoform, for example, Ik-2. The ZF embryo animal model carrying the Ik-2 transgene can be used to screen and identify agents that interfere with or overcome

normal Ik-2 function, for example, inducing B- or T-cell cancers, particularly leukemia. Potential cancer-inducing agents such as proteins, gene alterations, pharmaceuticals, toxins, and the like, are screened by administration to the model, and the disruption of normal function is monitored. An Ik-2 transgenic ZF embryo  
5 model thus can provide a screening assay for potential carcinogens.

In an alternative embodiment of the invention, the ZF embryo is transformed with a non-DNA binding form of Ikaros. The non-DNA binding form can be, for example, Ik- 4, 5, 6, 7, 8, 9, or 10, each of which lacks the three N-terminal zinc fingers required to confer high affinity DNA binding. A mutant Ikaros  
10 protein can also be used, for example those Ik deletion and insertion mutants described in PCT Patent Application PCT/US99/26274 and discussed more fully below. Because the ZF model containing a non-DNA binding Ik, e.g. Ik-4, develops leukemia at later stages of development, it can be used to screen for preventative and therapeutic agents.

15

### **Brief Description Of The Figures**

Figures 1A1 - 1D3 are computer generated images demonstrating successful microinjection and expression of human Ikaros proteins in Zebrafish embryos.

Figures 1A2, and 1A3 show a gfp-positive ZF embryo at mid gastrula  
20 stage, 6 hpf (Fig. 1A1), and Ikaros cDNA (Fig. 1A2) and control beta actin cDNA (Fig. 1A3) amplified from embryonic mRNA.

Figures 1B1-1B4 show a gfp-positive ZF embryo at prim-5 stage, 24 hpf (Fig. 1B1), and Ikaros cDNA (Fig. 1B2) and control beta actin cDNA (Fig. 1B3, 1B4) amplified from embryonic mRNA.

Figures 1C1-1C3 show a gfp-positive ZF embryo at prim-5 stage, 24 hpf (Fig. 1C1), and Ikaros cDNA (Fig. 1C2) and control beta actin cDNA (Fig. 1C3) amplified from embryonic mRNA.

Figures 1D1-1D3 show a gfp-positive ZF embryo at long-pec stage, 48 hpf (Fig. 1D1), and Ikaros cDNA (Fig. 1D2) and control beta actin cDNA (Fig. 1D3) amplified from embryonic mRNA.

Figures 2A-2F are computerized photographic images demonstrating Ik-4 and Ik-2 transgene expression in ZF embryos by whole-mount *in situ* hybridization.

Figure 2A shows a control embryo at 17 hpf with a negative hybridization result using the Ik-4 rhyboprobe.

Figures 2B-2D show Ik-4 injected embryos at 17 hpf, each demonstrating positive hybridization result with Ik-4 expression in the trunk region of the embryo. Figures 2E-2F are Ik-2 injected embryos at approximately 17 hpf, demonstrating positive hybridization with Ik-2 expression in the trunk region of the embryo.

Figure 3A is a computerized photograph of a medium sagattale section of the trunk region of a 48 hpf ZF embryo showing anterior to the left and dorsal to the top of the frame and providing a layout of the "dorsal" hematopoietic site.

Figures 3B-3E show frozen sagattal sections of the embryo immuno stained with antibodies raised against human Ikaros.

Figure 3B demonstrates Ik-4 immuno staining localized to the nuclei and cytoplasm in circulating blood cells, while Figure 3C demonstrates Ik-4 immuno staining in the mesenchymal hematopoietic cells of the dorsal aorta ventral wall.

Figure 3D demonstrates Ik-2 immuno staining localized to the nuclei in cytoplasm of circulating blood cells. Specificity of the anti-Ikaros antibodies to the human protein was confirmed by control staining. In non-injected ZF embryos, no cross reactivity with the endogenous ZF Ikaros was observed (Fig. 3E).

Figures 4A-4O are computerized photographic images showing expression patterns of the ZF early hematopoietic genes GATA-1, c-MYB, Rag-1, and Ick.

Figure 4A is a control embryo at 17 hpf with GATA-1 positive cells in the trunk region stained pink with fast red. No Ikaros staining is present.

Figure 4B is a lateral view of an Ik-4 transgenic embryo at 17 hpf showing blue-purple spots in patches marking Ik-4 expression and a strong pink staining of the ICM region.

Figure 4C is a lateral view of an Ik-2 transgenic embryo at 17 hpf showing blue-purple signals of the Ik-2 and no traces of GATA-1 expression.

Figure 4D is a laser confocal image of the embryo shown in Figure 4A, with fluorescent GATA-1 positive ICM staining.

Figure 4E is a laser confocal image of the embryo shown in Figure 4B, having a dramatic increase in the ICM due to abnormal expansion of GATA-1 positive cells.

Figure 4F is a laser confocal image of the embryo shown in Figure 4C, demonstrating a decline in GATA-1 expression.



Figure 4G is a laser confocal image of the control embryo at 15 hpf showing fluorescent c-MYB positive strip.

Figure 4H is a laser confocal image of the Ik-4 transgenic embryo at 15 hpf showing an expansion of the c-MYB positive cells in the cotal part of the ICM.

Figure 4I is a laser confocal image of the Ik-2 transgenic at 15 hpf with nearly total failure of c-MYB expression.

Figure 4J is a lateral view of the control 4 dpf ZF larvae with Rag-1 positive cells localized to the area of pharangyl arches between the eye and pectorial fin.

Figure 4K is a lateral view of the Ik-4 injected larvae at 4 dpf with Rag-1 positive thymic site.

Figure 4L is a lateral view of the Ik-2 injected larvae at 4 dpf with Rag-1 positive thymic site.

Figure 4M is a control non-injected ZF larvae at 4 dpf with lck positive thymic site.

Figure 4N shows an Ik-4 injected ZF larvae at 4 dpf with lck positive thymic site.

Figure 4O shows an Ik-2 injected ZF larvae at 5 dpf without lck positive thymic site.

Figure 5A is a graphic representation of hematopoietic cell indexes calculated for adult ZF derived from Ik-4 injected embryos (n=18), adult ZF derived

from Ik-2 injected embryos (n=22), and adult ZF derived from control non-injected embryos (n=10).

Figures 5B-5C are computerized microscopic images of kidney hematopoietic cells imprinted onto slides from intact embryos and differentially  
5 stained with Wright/Giesma.

Figure 5B shows imprinted cells of ZF kidney derived from Ik-2 injected embryos.

Figure 5D shows kidney hematopoietic cells from ZF derived from Ik-4 injected embryos.

10 In Figures 5B, 5C, and 5D myeloid cells are marked as M, erythroblast cells are marked as E, and lymphoid cells are marked with arrows pointing to the cells.

### **Detailed Description of the Invention**

The instant invention relates to the discovery that expression of  
15 Ikaros isoforms in ZF embryos provides modulation of hematopoietic cell development. Such modification is specific, and correlates with development events in human hematopoietic cells and with specific hematopoietic disorders, including leukemias and anemias.

Accordingly, the transgenic ZF animal model described more fully in  
20 the examples below provides a rapid and efficient screening assay for agents that modulate normal hematopoietic cell development. The animal model is thus useful for studying and understanding regulation of hematopoietic, as well as for screening agents for prevention and/or therapeutic applications.

In a preferred method of the invention, a zebrafish embryo carrying a transgene encoding a DNA-binding or non-DNA binding Ikaros protein is provided for analysis. ZF embryos expressing DNA binding Ikaros protein are useful for studying normal B- and T- cell lineage development, and for screening agents suspected of altering normal development. In addition, this model can be used to screen agents effective to increase red blood cell counts, as an indication of useful anti-anemia therapy.

ZF embryos expressing non-DNA binding isoforms (Ik-4, 5, 6, 7, 8 and deletion or insertion Ik mutants) can be used to screen for potential therapeutic candidates, particularly for the prevention and/or treatment of hematopoietic disease. Examples of mutant Ikaros proteins include those lacking the following amino acid sequence: KSSMPQKFLG [SEQ ID NO: 5]. An exemplary insertion mutant is an Ikaros protein containing an insertion of the following amino acid sequence: VTVGADDFRDFHAIIPKSFSR [SEQ ID NO 6]

15

### Examples

The invention may be better understood with reference to the following Examples. These are intended only to exemplify the invention, and not to limit the scope of the invention in any way.

### **Example 1**

20

#### Expression Constructs and Microinjection of Zebrafish Embryos

**Fish and embryos.** The adult wild type ZF were maintained generally according to the Zebrafish book recommendations (Westerfield, 1995). Males and females were kept in 10 G tanks, 70 fish per tank, with a constant slow flow of conditioned water at 26°C and a controlled 14 hours day/10 hours night

cycle. Embryos were obtained through natural spawning in breeding cages with a netted false bottom or by *in vitro* fertilization using eggs and milt collected from the mature females and males anesthetized with tricaine (Sigma). Embryos were kept at 28.5 °C in Petry dishes, 30-50 per dish.

5                   Five days after hatching, fry were transferred to a nursery for 2 weeks and raised in 1 G mouse cages at 28.5°C. Larvae were fed with live food, paramecia and brine shrimps, according to recommendations of Dr. Stephen Ekker (personal communication). The survival rate was over 95%. Juvenile ZF were transferred to 10 G tanks, treated as adult fish as described above and raised to  
10 maturity for another 3 months. For *in situ* hybridization studies, embryos at 1-5 days post fertilization (dpf) were treated with 0.003% phenylthiourea (Sigma) to prevent pigmentation.

**Genes and expression vectors.** Transgenic expression of human Ikaros isoforms Ik-2 and Ik-4 were driven by the promoter/enhancer region of carp  
15  $\beta$ -actin gene in all-fish expression vector pFV4aCAT (Caldovic and Hackett, 1995). The DNA vectors were constructed by cloning the human wild type Ikaros cDNA Ik-2 and Ik-4 into pFV4aCAT at the NotI and SpeI sites. The vectors were re-named hIK4wt pFV4aCAT and hIK2wt pFV4aCAT, respectively. After further digestion  
with XbaI, the linearized fragments containing Ikaros cDNA were then purified with  
20 a Spin X column (Costar) and used for microinjections.

**Microinjections.** Microinjections were performed with the help of a SMZ-10A stereo microscope (Nikon) and Transjector 5246 (Eppendorf) at room temperature (RT) using glass micropipettes with splinted sharp tips of 2-3  $\mu$ m

diameter. The eggs in chorions at the early one-cell stage were positioned in grooves of agar-lined Petri dishes, as described in Westerfield, 1995.

The constructs were coinjected, according to the method recommended by Hyatt and Ekker, 1999, with green fluorescent protein (GFP) mRNA (147.4 ng/ $\mu$ L), to confirm the presence of the injected molecules and to enable selection of GFP-positive fish for future analyses. DNA was dissolved to a final concentration of 10 ng/ $\mu$ L in Hank's saline containing 0.01 % phenol red, in order to follow the injection procedure. Approximately 5 nl of the injected medium containing the equivalent of approximately 50 pg ( $10^7$  copies) of the construct was injected into the cytoplasm of the egg's blastodisk under visual control. Each construct was injected independently in 3-5 series of injections and the data obtained was pooled. Following the injections, eggs were incubated at 28.5°C.

Embryo observations were carried out with a SMZ-10A stereo microscope (Nikon), equipped with an additional filter setting for fluorescence detection and a specially designed transparent heating tray to keep embryos at constant temperature. Pictures of the embryos were taken with a H-III Photomicrographic System (Nikon) on Ektachrome 100X film (Kodak). Fluorescent embryos were imaged using MRC-1024 Laser Scanning Confocal Imaging System (Bio-Rad). The embryos and larvae were observed and analyzed at 6, 24, and 48 hours post fertilization (hpf) and at 3, 4, and 5 days post fertilization (dpf), or were raised to adulthood for future analyses (see above).

**mRNA Isolation and RT-PCR.** mRNA was extracted from individual zebrafish embryos and adult fish tissues using the Oligotex<sup>TM</sup> Direct mRNA isolation Kit (Qiagen, Valencia, CA). Possible DNA contamination was

eliminated by incubating all mRNA with 1 µl DNase (Promega, RQ1 RNase Free DNase) in 100 µl final volume of buffer containing 50 mM NaCl, 5mM Tris-Cl, pH7.5 at 37°C for 30 minutes. The reactions were stopped by phenol/chloroform extraction and mRNA was precipitated with ethanol. Reverse transcription was  
5 done with a 500 ng mRNA template in the 20 µl final volume using Advantage™ RT-for-PCR Kit (Clontech). The cDNA obtained by reverse transcription was diluted to a total volume of 100 µl by adding 80 µl H<sub>2</sub>O. PCR amplification of Ikaros cDNA was achieved using 10 µl of the diluted cDNA as template and the Advantage® cDNA Polymerase Mix (Clontech) in a 50 µl reaction volume. Primers  
10 for amplification of Ikaros cDNA are shown below:

F1, 5'-ATGGATGCTGACGAGGGTCAAGAC-3' [SEQ ID NO: 1] and  
R1, 5'-CTAGTGGAATGTGTGCTCCCCTCG-3' [SEQ ID NO: 2].

15 The integrity of the mRNA and cDNA was confirmed by PCR amplification of zebrafish β-actin cDNA in the same reaction with primers specific for β-actin:

20 ACTf, 5'-GATGATGCCCCCTCGTGCTGTTTTC-3' [SEQ ID NO: 3] and  
ACTr, 5'-TTTCTCTTTCGGCTGTGGTGGTGA-3' [SEQ ID NO: 4].

The 4 kb injected DNA fragment was used as a positive control PCR template for comparison to the size of the amplified fragments.

**Sectioning.** Dechorionated or hatched ZF embryos at 48 hpf were fixed in 4% paraformaldehyde/phosphate buffer (PBS) at 4°C for 2 hours, soaked in 30% sucrose, embedded into OCT cryostat embedding medium (Fisher) and frozen in liquid N<sub>2</sub> exactly as described previously in Westerfield, 1995. Sections of 5 µm were prepared with the cryotome CM 1800 (Lieca). Slides with mounted ZF sections were stored at - 80°C until rehydrated by washing 3 times with PBS, pH 7.4 (Celox Laboratories). Sections were stained with hematoxyline and eosine as described in Luna, 1968, or subjected to immunostaining.

**Immunostaining.** Tissue sections were permeabilized by soaking with a blocking solution of PBS containing 2.5% bovine serum albumin (BSA) (Sigma) and 0.1% Triton-X-100 (Fisher Biotech) for 30 minutes, and then treated with primary antibodies (rabbit anti-Ikaros IgG (1:100), Parker Hughes Institute) for 1 hour at room temperature. Treated sections were washed 3 times with PBS, stained with fluorescein isothiocyanate-labeled secondary antibodies (donkey anti-rabbit Ig (1:40), Amersham) for 1 hour at room temperature and washed 3 times with PBS, after which Vectashield mounting medium with propidium iodide (Vector Laboratories) and coverslips were applied. Stained ZF embryo sections were imaged using MRC-1024 Laser Scanning Confocal Imaging System (Bio-Rad).

**Whole-mount *in situ* hybridizations** were carried out on embryos at 15 - 19 hpf and 4, 5 dpf according to the methods described in Jowett, 1999. Ikaros riboprobes were labeled with digoxigenin, whereas GATA-1, c-MYB, Rag-1, and lck probes, used in two-color/fluorescent *in situ* hybridizations, were labeled with fluorescein. Synthesis of RNA probes was performed by *in vitro* transcription using a DIG RNA Labeling Kit (Roche Molecular Biochemicals). For Ikaros riboprobes,

human Ikaros cDNA (Ik-4 or Ik-2) used as a template was cloned to the pBluescript/KS+ vector and linearized at the XhoI site. Anti-sense RNA was *in vitro* transcribed with T7 RNA polymerase for 2 hours at 37°C and labeled by digoxigenin-11-UTP added to the nucleotide mixture. GATA-1 template cloned to the pBluescript/SK+ vector was linearized with XbaI. c-MYB or Ick cloned to pBKCMV were linearized with EcoRI, and Rag 1 cloned to pCR 2.1 vector was linearized with Hind III restriction endonuclease.

Anti-sense probes were synthesized with T7 RNA polymerase, as above. Sense probes used for control staining were synthesized with T3 RNA polymerase. Prior to *in situ* hybridization, the efficacy of reaction was confirmed by gel electrophoresis and Northern hybridization. Embryos fixed with 4% paraformaldehyde for 12 hours at 4°C were gradually dehydrated in methanol and kept at -20°C overnight. Following rehydration, embryos were prehybridized for 5 hours at 70°C, then hybridized with corresponding riboprobe(s) at 70°C (overnight) and finally treated with anti-DIG or anti-fluorescein Fab fragments for immunolocalization of the haptens. Detection of the DIG antibody-alkaline phosphatase conjugate was carried out by staining with nitroblue tetrazoleum / 5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP) substrate mixture which produces an insoluble, blue-purple precipitate. Visualization of fluorescein antibodies conjugated to alkaline phosphatase complex was accomplished by staining with Fast Red (Boehringer Mannheim) which produces a precipitate that is both chromogenic (pink) and fluorescent. After hybridization, embryos were re-fixed in 4% paraformaldehyde and photographed. Images of embryos were then taken with an H-III Photomicrographic System (Nikon) on Ektachrome 100X film (Kodak).



Fluorescence in the embryos was detected and imaged using MRC-1024 Laser Scanning Confocal Imaging System (Bio-Rad) mounted on a Nikon Eclipse E800 upright microscope with high numerical aperture objectives. Digital data from 30-34 optical section series were collected and 3D images were reconstructed using Laserssharp software (Bio-Rad, Hercules CA) and printed on a Fuji Pictography thermal transfer printer (Fuji, Elmsford, NY).

**Adult fish, tissues, and kidney imprints.** The size, color, sex and weight of adult three-month-old fish were determined. Blood was collected from the caudal vein from anesthetized fish and organs/tissues (spleens, intestines, brains, eyes, hearts, and kidneys) were dissected and frozen in liquid Nitrogen for RT-PCR analysis. Prior to freezing, color and weight of spleens and livers were analyzed and weight indexes were calculated (weight of the organ x 100/total fish weight). Kidneys were imprinted on slides which were air dried and stained with Wright/Giemsa according to the University of Maryland Special Hematology Laboratory protocols. Kidney imprints were studied microscopically for cellular composition and photographed using a microscope Eclipse E800 (Nikon) and a H-III Photomicrographic System (Nikon).

**Cell morphology analysis.** Blood cells were typed according to cell morphology and PAS, Sudan black, and myeloperoxidase staining. Cell counts in the kidney imprints were performed at x100 magnification using an eye-piece grid in 10 different areas of each sample. The number of lymphoid, myeloid, and erythroid cells as well as granulocytes and monocytes was determined and cell indexes were calculated for each of the cell lineages.

**Statistics.** Data obtained from adult fish measurements and from kidney cell counts were checked for normal distribution and subjected to a standard Student's two-tailed test with Welch's correction, when necessary. Statistical analysis and graphing were performed using GraphPad Prism version 2.0 (GraphPad Software, Inc., San Diego, CA).

### Example 2

#### Transgenic Expression of Human Ikaros Isoforms Ik-4 and Ik-2 in ZF Embryos

Linearized expression vectors hIK4wt pFV4aCAT and hIK2wt pFV4aCAT were mixed with GFP mRNA and microinjected into one-cell stage ZF embryos to force expression of the dominant-negative human Ikaros isoform Ik-4 and the DNA binding human Ikaros isoform Ik-2 (control) during primitive hematopoiesis. The microinjections were successful in >95% of all embryos, as evidenced by a strong green fluorescence documenting the expression of the coinjected GFP mRNA from mid-gastrula until prim-5 stage (Figures 1A1, 2A1,3A1).

Total mRNA was extracted from GFP-positive individual ZF embryos at 6 hpf, mid-gastrula stage (Figure 1A1), at 24 hpf, prim-5 stage (Figures 1B1, 1C1) and at 48 hpf, Long-pec stage (Figure 1D1). The extracted mRNA was reverse-transcribed using oligo-dT and random hexamers. The resulting cDNAs were amplified with ZF  $\beta$ -actin primers (Figures 1A3, 1B3, 1C3, and 1D3) to test the integrity of the extracted mRNAs. Human Ikaros expression was analyzed by amplification with Ikaros-specific primers hIKEX7R1 and hIKEXIF (Figures 1A2, 1B2, 1C2, and 1D2).

RT-PCR analysis of total RNA from GFP-positive embryos (n=13-18

per time point) confirmed the time-dependent expression of human Ik-4 and Ik-2 mRNA. At late blastula stage (4 hpf), no human Ikaros transgene expression had been detected, likely due to a lack of transcriptional activity of the zygotic genes  
5 (data not shown). In contrast, during embryonic shield formation at mid-gastrula stage (6 hpf), prim-5 stage (24 hpf), and long-pec stage (48 hpf), 100 % of the tested embryos expressed the corresponding human Ikaros transgene (1A2, 1B2, and 1C2, respectively).

Expression of the human Ikaros transgenes Ik-4 and Ik-2 was  
10 transient. Only half of the hatching embryos (72 hpf), one third of the larvae (96 hpf), and none of the adult ZF tissues showed RT-PCR evidence for human Ikaros transgene expression (data not shown). Microinjections did not significantly affect the viability and survival rate of the ZF embryos. Of 162 non-injected control embryos, 146 (90.1%) developed up to hatching without any visual abnormalities.  
15 Similarly, 316 of 361 (87.5%) GFP-positive embryos microinjected with the Ik-4 expression vector hIk4wtpFV4aCAT and 256 of 303 (84.5%) GFP-positive embryos microinjected with the Ik-2 expression vector hIk2wtpFV4aCAT developed normally (Table 1).

Table 1

## Survial Rates and Embryonic Development following Ikaros injections

Construct	# Injected Fertilized Eggs (n)	# GFP-Positive Embryos at 3-6 hpf	# Normal Embryos 3-48 hpf* n (%)	# Normal Larval 72-96 hpf (n)	# Oligochromen Larval 72-96 hpf n (%)
Ik4	379	361	316 (87.5%)	183	27 (13%)
Ik2wt	312	303	256 (84.5%)	235	11 (4.5%)
act control	162	0	146 (90.1%)	134	5 (3.7%)

\* including embryos taken for analysis

A lack of pigmentation (a/oligochromemia) in the circulating blood cells as seen through pericardium, was observed at 48-72 hpf in 27 out of 183 (13%) Ik-4 injected embryos and fry. This data is contrasted with a/oligochromemia observed in only 4.5% and 3.7% of the Ik-2 injected embryos and intact control, respectively.

## Example 3

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*in situ* Localization of nIkaros transgenes

The topographical profile of the human Ikaros transgenes Ik-4 and Ik-2 expressed in the ZF embryos at 17-19 hpf was confirmed by whole-mount *in situ* hybridization using digoxigenin-labeled Ikaros riboprobes. Probes hybridized to the human Ikaros mRNA were immunolocalized with anti-DIG Fab fragments and detected by chromogenic reaction with NBT/BCIP. No false positive signals were

detected in non-transgenic control ZF embryos (Figure 2A). In transgenic ZF embryos, the chromogenic (blue-purple) signal of human Ik-4 or Ik-2 transgene expression was largely localized to the trunk region containing the intermediate cell mass (ICM) where primitive hematopoiesis takes place (Figures 2B-2F), reminiscent of the expression profile of other regulators of hematopoiesis such as GATA-1 and c-MYB (Detrich et al., 1995, Amatruda and Zon, 1999).

At 48 hpf, embryonic hematopoiesis in the ZF shifts from the ICM to the dorsal mesentery and forms the "dorsal" fetal hematopoietic site (Detrich et al., 1995, Amatruda and Zon, 1999). At this transition stage from primitive to definitive hematopoiesis, the trunk axial vessel complex, i.e., dorsal aorta and axial vein, stretches along the anterior-posterior axis, between the notochord and trunk endoderm, and contains circulating embryonic blood cells (Figure 3A). ZF embryos derived from the Ik-4 injected and from the Ik-2 injected eggs were fixed with paraformaldehyde at 48 hpf and frozen sagittal section were stained with hematoxylin/eosin or immunostained with the antibodies raised against human Ikaros. Sections were examined by laser confocal microscopy. The median sagittal section of the trunk region (A) of the 48 hpf ZF embryo with anterior to the left and dorsal to the top of the frame, provides a layout of the "dorsal" hematopoietic site with dorsal aorta (DA) and axial vein (AV) with mesenchyme hematopoietic cells of the dorsal aorta ventral wall (M) and circulating primitive blood cells (PB). From the dorsal side the axial major vessel complex are bordered by the notochord (NC) and neural tube (NT); from the ventral side the vessels are neighbored by the endoderm (E) and the yolk extension (YE).

Examination of the sagittal sections of the trunk region in Ikaros transgenic ZF embryos by immunofluorescence staining with antibodies directed against human Ikaros and confocal laser scanning microscopy showed expression of human Ikaros in circulating lymphohematopoietic cells (Figure 3B) as well as in the cells of the ventral wall of dorsal aorta (Figure 3C) and ventral vein region (data not shown). Human Ik-4 isoform showed cytoplasmic and patchy nuclear expression in hematopoietic cells of transgenic ZF embryos (Figures 3B-3C), reminiscent of its intracellular localization pattern in human cells (Sun et al., 1999). A similar pattern of subcellular compartmentalization was observed in circulating ZF blood cells expressing the Ik-2 isoform. Ik-2 protein was detected in the nuclei and cytoplasm of the blood cells (Figure 3D). No false positive signals were detected in non-transgenic control ZF embryos (Figure 3E).

#### Example 4

##### Deregulated expression of GATA-1, c-MYB and *lck* Genes in Human Ik-4 and Ik-2 transgenic ZF Embryos

The impact of Ik-4 and Ik-2 transgene expression in the ZF embryos on the expression pattern of the early hematopoietic and lymphopoietic markers GATA-1, c-MYB, Rag-1, and *lck* was evaluated. Ik-4 and Ik-2 transgene expression interferes with the normal expression of ZF GATA-1, c-MYB, and *lck*, but not with Rag-1. The zinc-finger transcription factor GATA-1 is one of the central regulators in hematopoietic cell differentiation within the myeloid and erythroid lineages (as thoroughly reviewed by Orkin and Zon, 1997). The transcriptional regulator of myelopoiesis encoded by the proto-oncogene c-MYB and its target *c-myc* have been implicated in myeloid leukemogenesis, as reviewed by Wolff, 1996 and Weston,

1999. Both GATA-1 and c-MYB are expressed in the ZF during 15-24 hpf and they strongly demarcate the forming ICM, the earliest site of primitive hematopoiesis (Detrich et al., 1995, Liao et al., 1998, Bahary and Zon, 1998).

By comparison, expression of the lymphoid marker Rag-1 in the ZF commences at 3 dpf, when thymocyte precursors seed the bi-lateral thymic anlage (Trede and Zon, 1998). A similar pattern of expression restricted to bilateral thymi was shown for *lck* (Dr. Nikolaus Trede, personal communication).

Expression patterns of the ZF early hematopoietic genes in the presence of Ik-4 and Ik-2 transgene expression were studied in the ZF embryos by means of two color/fluorescence whole-mount *in situ* hybridization. All images are positioned anterior to the top and dorsal side to the right of the frame.

In contrast to the first two markers, Rag-1 expression is restricted to thymocytes after they seed the thymus anlagae at 3 dpf. We observed Rag-1 expression in the bi-lateral thymi at 4 dpf in all tested fish from the Ik-4 injected and Ik-2 injected groups and in the control with no regard to the transgene expression. Rag-1 was transcribed bi-laterally in the location of thymus primordial.

Following two color/fluorescence *in situ* hybridization with human Ikaros and ZF GATA-1 riboprobes, all non-injected control embryos at 17 hpf were found positive for GATA-1 expression and showed no false positive signals of human Ikaros expression (Figure 4A). GATA-1 positive cells formed a distinct strip of the ICM in the trunk region between the somite mesoderm and the yolk protrusion, which was remarkably vivid with the use of fluorescence (Figure 4D). Notably, in the ZF embryos expressing the human Ik-4 transgene, the GATA-1

positive ICM region was much larger than in non-injected control embryos (Figures 4B & 4E). In contrast, in the ZF embryos expressing the Ik-2 transgene, the GATA-1 positive cells in the ICM site were few and formed a dotted rather than a solid line (Figures 4C & 4F).

5 In all control embryos tested with human Ikaros and ZF c-MYB riboprobes at 15 hpf, c-MYB-positive cells were condensed in the distinct region of the ICM (Figure 4G). Similar to GATA-1, the region of c-MYB positive cells was visibly enlarged in the Ik-4 transgenic ZF embryos (Figure 4H), and dramatically decreased in the Ik-2 transgenic embryos with expression restricted to the uttermost  
10 caudal portion of the ICM (Figure 4I).

In contrast, expression of Rag-1 was not altered in the Ik-4 nor in the Ik-2 transgenic embryos. In all embryos, positive fluorescent signals which mark Rag-1 expression were restricted at 4 dpf to the bi-lateral sites of developing thymus, and were localized to the region of pharyngeal arches between the eye and pectoral  
15 fin (Figures 4J, 4J, 4L). Similar to Rag-1, the ZF lck expression in the control non-injected ZF larvae at 4-5 dpf was restricted to the thymic location (Figure 4M). The lck expression was not affected in the Ik-4 injected larvae (Figure 4N) but was dramatically decreased or totally absent in the Ik-2 injected ZF larvae at 4-5 dpf (Figure 4O).

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### Example 5

#### **Abnormal hematopoiesis with Lymphoid Hyperplasia in Adult ZF Derived from Ikaros 4 Transgenic Embryos**

Adult fish derived from the Ik-4 and Ik-2 transgenic embryos as well as from non-injected control embryos were raised in similar conditions as 3 separate



groups. At 3 months of age, these fish were all in apparent good health with normal shape and coloration, and reached maturity according to the breeding behavior and pair-wise mating. The body weight, body length, liver size and spleen size of Ik-4 and Ik-2 injected ZF were not different from those of the adult non-transgenic control fish (Table 2).

Table 2

## Analysis of Adult Fish

	n	Mean( $\pm$ SEM)	Median	Range	p-value	Difference
10 Length						
Ik-4	18	3.74 $\pm$ 0.07	3.8	3.15-4.10	0.48	NO
Ik-2	22	3.53 $\pm$ 0.05	3.5	3.0-4.0	0.18	NO
intact control	8	3.76 $\pm$ 0.22	3.95	2.75-4.5		
15 Weight						
Ik-4	18	491.71 $\pm$ 32.4	531.25	274.92-704.29	0.28	NO
Ik-2	22	416.87 $\pm$ 30.32	367.49	272.28-862.7	0.28	NO
intact control	8	454.19 $\pm$ 52.59	482.52	181.68-651.39		
Liver/total wt						
20 Ik-4	18	2.36 $\pm$ 0.32	1.57	1-4.98	0.29	NO
Ik-2	22	2.06 $\pm$ 0.32	1.57	0.65-6.56	0.43	NO
intact control	8	2.13 $\pm$ 0.27	2.25	0.89-3.1		
Spleen/total wt						
Ik-4	17	0.08 $\pm$ 0.01	0.08	0.03-0.17	0.35	NO
25 Ik-2	19	0.11 $\pm$ 0.01	0.11	0.03-0.21	0.15	NO
intact control	6	0.09 $\pm$ 0.02	0.1	0.04-0.13		

In adult ZF, kidney plays the role of the bone marrow in mammals.

Kidney hematopoietic cells from 50 mature 3-month-old adult ZF derived from the Ik-4 transgenic, Ik-2 transgenic, and intact embryos were imprinted onto slides and differentially stained with Wright/Giemsa for microscopic examination of cellularity and cellular composition. Hematopoietic cell indexes were calculated for 18 adult ZF derived from the Ik-4 injected embryos, 22 adult ZF derived from the Ik-2 injected embryos, and 10 adult ZF derived from the control non-injected embryos.

To examine representation of hematopoietic cell types in adult fish, kidneys extracted from 18 Ik-4 injected, 22 Ik-2 injected and 10 non-injected adult ZF were imprinted on slides and stained differentially with Wright/Giemsa. All samples contained a multilineage population of hematopoietic cells including both progenitors and mature forms (Table 2, Figure 5A).

Imprints from the control, non-injected ZF showed multilineage hematopoiesis with marked myeloid (43%) and erythroid (30%) hyperplasia. Other cell types identified in the imprints consisted of lymphoid cells (15%), granulocytes (10%) and monocytes (5%) (Table 2, Figures 5A - 5B). In contrast, in the ZF derived from the Ik-4 injected embryos, lymphoid cells represented 49% of hematopoietic cell population, whereas myeloid and erythroid cells as well as granulocytes were reduced in numbers (24%, 21%, and 3%, respectively), and the number of monocytes remained unchanged (3%) (Table 2, Figures 5A & 5D). In the ZF derived from the Ik-2 injected embryos, the number of erythroid cells was drastically increased (46%), while cells of the myeloid and lymphoid lineages were present in reduced numbers (29% and 8%, respectively). The number of

granulocytes was slightly increased (13%), and the number of monocytes was not markedly changed (4%) (Table 2, Figures 5A & 5C).

The data described herein provides evidence that the human leukemogenic dominant-negative isoform Ik-4 and human DNA-binding isoform Ik-2 are transiently expressed in ZF embryos during primitive and "fetal" definitive hematopoiesis. Early ZF hematopoietic transcription factors GATA-1 and c-MYB are affected by Ik-4 and Ik-2 expression in opposite ways; they are upregulated by Ik-4 and downregulated by Ik-2 expression.

In adult kidney, distinct lymphoproliferative disorder was observed in ZF derived from the Ik-4 transgenic embryos, and erythroproliferative disorder was detected in ZF derived from the Ik-2 transgenic embryos. The observed phenomenon can be described as a hyperplasia of specific blood cell types which occurs at the expense of other cell lineages as a late response to the Ik-4 and Ik-2 transgene expression during embryonic hematopoiesis. Accordingly, the data supports the transgenic ZF as a useful experimental model to study leukemogenesis, Lymphoproliferative disorders (e.g. leukemias) and erythroproliferative disorders (e.g. anemia).

## DISCUSSION

All vertebrates including mammals and fish, utilize the same basic principles and share the same major steps of blood development, with waves of primitive and definitive hematopoiesis, successive changes of hematopoietic sites in ontogeny, and colonization of hemopoietic organs by blood cell precursors of specific lineages. In this respect, the ZF provides an excellent model to define genes

and genetic pathways essential for blood cell differentiation and development of hematopoietic disorders.

In several large-scale chemical mutagenesis screens, over 50 mutations were identified in the ZF which affect differentiation in red cell (Ransom et al., 1996, Weinstein et al., 1996) and white cell lineages (Trede and Zon, 1998, Dr. Nikolaus Trede, personal communication). It has been shown recently that disruption of the *sau* gene, which leads to microcytic hypochromic anemia phenotype in ZF corresponds to impaired gene coding for erythroid-specific d-aminolevulinate synthase (ALAS2/ALAS-E) necessary for heme biosynthesis, and results in congenital sideroblastic anemia in humans (Brownlie et al., 1998). Mutation of another gene, *yqetp61*, leads in the ZF to a photosensitive porphyria. This was linked to uroporphyrinogen decarboxylase (UROD)-deficiency which causes hepatoerythropietic porphyria in humans (Wang et al., 1998). A spontaneous blood mutation, *cloche* (*clo*), was found to affect both blood and endothelial cell differentiation in ZF (Stainier et al., 1995), most probably by disrupting normal SCL (Tal-1) expression in hemangioblasts (Liao et al., 1998, Gering et al., 1998). These findings demonstrate that at least some ZF blood mutations serve as models for human blood disorders.

In support of the ZF as a model animal, it is noted that feasible and reliable vehicles necessary for either transient transgene expression or stable integration and expression are well developed for the ZF. In addition, the data recited herein demonstrates that two isoforms of the human Ikaros gene that play a critical role in lymphocyte differentiation were expressed in the ZF during embryonic hematopoiesis. Transgene expression was regulated in the ZF cells, for it

started after the onset of the zygotic genome transcription. It persisted for the first two days in 100% of the injected embryos and in approximately 50% and 40% of the 3 dpf embryos and 4 dpf larvae, respectively.

The  $\beta$ -actin promoter, cloned into the all-fish expression cassette have used herein was intended to drive transgene expression in all types of fish cells. Expression was detected in a variety of cells including mesenchyme cells of dorsal aorta ventral wall and ventral vein region as well as in circulating blood cells. Thus, transgene activity in the appropriate cells with appropriate micro-environment may be the cause of future hematopoietic alteration.

A layout of blood development in the ZF embryo served as a necessary background for the present study. In the ZF, the first cells committed to blood differentiation were defined as early as the end of gastrulation as two lateral stripes of ventral mesoderm with cells (hemangioblasts) expressing, as shown by *in situ* hybridization, early hematopoietic and vasculogenic markers SCL, GATA-1, GATA-2, c-MYB and LMO2 (Detrich et al., 1995, Gering et al., 1998, Liao et al., 1998, Thompson et al., 1998, Amatruda and Zon, 1999). These cells migrate to somite mesoderm to form in about 2 hours (the 5 somite stage) the intermediate cell mass (ICM). The ICM is known to be the site of primitive hematopoiesis in fish (Al-Adhami and Kunz, 1977, Detrich et al., 1995; Willett et al., 1999) and comprises hematopoietic (primarily embryonic erythroblasts), vasculogenic cells as well as pronephric cell precursors (Zon, 1995, Weinstein et al., 1996, Liao et al., 1998, Thompson et al., 1998, Willett et al., 1999). The ICM declines with the production of circulating erythroblasts and erythrocytes and by 30 hpf hematopoiesis shifts to the nascent "dorsal" site possibly the first sites of definitive hematopoiesis, the

dorsal aorta and to the "ventral vein region" containing blood cell precursors in the axial vein walls and surrounding mesenchyme (Liao et al., 1998, Thompson et al., 1998, Willett et al., 1999). From here two separate seedings take place: of thymus (at 65 hpf) and of kidney primordia (starting at 96 hpf) (Hansen and Zapata, 1998, Trede and Zon, 1998, Willett et al., 1999). While thymus is colonized by T-lymphocyte precursors, pronephros is seeded with different lineage progenitors including erythro-, myelo- and B-lymphocytes. Finally, with kidney differentiation into head kidney (pronephros) and trunk kidney (mesonephros), the main multilineage hematopoietic site in adults is formed which is unequivocally considered to be a bone marrow equivalent (Rowley et al., 1988, Hansen and Zapata, 1998).

#### **Interaction of the human Ikaros isoforms with the ZF hematopoietic genes.**

The data presented herein shows that the area of ZF GATA-1 expression in the Ik-4-positive embryos at 17 hpf was markedly increased than in the control and Ik-4 negative embryos. Taking into consideration that GATA-1 is the earliest marker to be expressed in blood cell progenitors, this shift in GATA-1 expression pattern suggests enlargement of the whole ICM region in the embryo. The effect of the Ik-2 transgene was totally opposite - the GATA-1 positive ICM area was drastically reduced. Similarly, human Ikaros isoforms affected expression of the ZF c-MYB. In the Ik-4 transgenic embryos at 15 hpf, c-MYB-positive area in the ICM was enlarged whereas in the Ik-2 transgenic embryos, the strip of c-MYB-positive cells was mostly missing showing a decline in c-MYB expression. Finally, in Ik-2 injected ZF larvae at 4 and 5 dpf, lck expression in thymocytes was visibly reduced or totally blocked. It should be noted that in ZF embryos, expression of ZF

*Ikaros* gene was detected by *in situ* hybridization in the ICM (at 5 somite stage and at 24 hpf) and then in the dorsal aorta at 46 hpf (Kawasaki et al., 1998). Thus, in addition to a role in determination of hematopoietic stem cell commitment to lymphoid lineage in adults (Hansen et al., 1997), *Ikaros* marks in the ZF embryo, the earliest hematopoietic lymphoid progenitors.

In humans and fish (trout), *Ikaros* was found to be highly conserved showing 75% homology in amino acid sequence and 92-98% identity in the active sites of the protein (Hansen et al., 1997). Structural similarity gives grounds to assume that both human *Ikaros* isoforms can interact with endogenous ZF *Ikaros*, as well as with other ZF genes involved in blood cell differentiation. Overexpression of DNA-binding *Ikaros* isoform in the blood cell progenitors as well as the occurrence of the non-binding isoform equally affects blood development.

#### **Distribution of transgene expression.**

*Ik-4* and *Ik-2* transgenes were expressed in the ZF embryos in a mosaic fashion. As evidenced by whole-mount *in situ* hybridization, human *Ikaros* RNA resided in various regions of the embryo but most commonly in the trunk area, in close proximity to the ICM site. Transgene expression was confirmed by immunostaining and human *Ikaros* isoforms were localized in the 48 embryos to a number of hematopoietic and nonhematopoietic cells. Along with the sites of ectopic expression, the trunk region of the embryo which at this stage contains the dorsal aorta and axial vein complex was commonly found positive also.

Human *Ik-4* isoform was detected in the circulating blood cells, as well as in the hematopoietic cells of dorsal aorta ventral wall and cells surrounding the caudal portion of the axial vein, known as a ventral hematopoietic site (Liao et

al., 1998). Human Ik-2 isoform was detected in circulating blood cells as well as in endothelial cells of the axial vessels. Large hematopoietic cells of the "dorsal" site as well as similar large cells in the blood stream retained Ik-4 protein in the nucleus and in the cytoplasm, whereas the smaller and much more round cells of primitive  
5 blood retained no signal in the nucleus supposedly due to its inactivation. Ik-2 protein was localized to the nucleus and to the cytoplasm of the circulating blood cells.

The data show that human Ikaros gene expression in the transgenic ZF embryo may be both ectopic and site-specific. While in non-hematopoietic cells  
10 Ik-4 and Ik-2 transgene activity is probably irrelevant to blood development, their action in the ICM and the "dorsal" sites of embryonic hematopoiesis may cause significant changes in the pattern of endogenous ZF Ikaros expression with dramatic consequences for blood cell differentiation. The observed changes in the GATA-1, c-MYB, and Ick expression patterns suggest that directly or indirectly the activity of  
15 these early blood cell markers was affected by the transgenes.

To avoid ectopic expression, transgenes may be targeted to specific cell types. Recent study of the ZF GATA-1 promoter showed that positive and negative *cis*-regulatory elements are essential for erythroid-specific expression (Meng et al., 1999). Promoters from lymphoid-specific ZF genes (Rag 1,2, Ick,  
20 Ikaros) can be used to force transgene expression exclusively in one of the cell lineages. By choosing a cell-specific gene promoter, transgene activation can be restricted to desired cell types.



**Primitive blood circulation.**

It was generally accepted that embryonic erythrocytes form the only population of circulating blood cells in the 24-48 hpf ZF embryos. However, large non-erythroid cells defined as granulocytes according to their ultrastructure, were found in the blood stream of the 48 hpf ZF embryos (Lieschke et al., 1999). This finding make it possible to assume that other types of blood cells, including lymphoid progenitors may be present in circulating blood in the ZF embryo. The data presented herein shows that in the 48 hpf transgenic ZF embryos, both Ik-4 and Ik-2 human Ikaros isoforms reside in the nuclei and cytoplasm of large non-differentiated cells found in circulation, as well as in the non-differentiated cells of the dorsal aorta ventral wall and ventral vein mesenchyme cells. These cells, affected by either dominant-negative Ik 4 expression or by overexpression of the DNA-binding Ik-2 isoform, may comprise the cell pool which seeds the kidney primordium, and thus, be responsible for future changes in adult hematopoiesis.

**15 Blood cell types in adult fish.**

Hematopoietic tissue in the ZF kidney is formed by cords of cells which surround blood vessels, in-between the renal tubules and glomeruli (Willett et al., 1999). Erythrocyte, granulocyte, lymphocyte, and monocyte differentiation has been reported in the adult fish kidney (Rowley et al., 1988) and with the exception of nucleated erythrocytes, the morphology of the other mature and differentiating hematopoietic cells closely resembles that of their mammalian counterparts. Morphological description of fish blood cells, including their ultrastructure and functions is summarized in Rowley et al., 1988.

There are two subpopulations of lymphocytes in fish with different immunological properties as reviewed by Miller et al., 1998, which precisely correspond to T- and B-cells. T-lymphocytes are located predominantly in thymus where Ikaros (Hansen and Zapata, 1998), Rag 1,2, and lck, a *src*-family protein  
5 tyrosine kinase implicated in T-cell maturation and activation (Trede and Zon, 1998) are expressed. B-lymphocytes are generated in the kidney; in trout and ZF, Ikaros (Hansen et al., 1997), Rag-1 and Rag-2 (Willett et al., 1997) and TdT (Hansen, 1997) expression in the pronephros was used to confirm the presence of pre-B-cells. A *tec*-family non-receptor tyrosine kinase expression was found recently in the ZF  
10 kidney (Haire et al., 1998) which may represent the *Btk* expression in the B-lymphocytes.

#### Neoplasia in fish.

For several reasons, neoplastic transformation in fish is not widely reported in the literature. Nonetheless, fish are susceptible to neoplasms and as  
15 models were successfully utilized in the studies of carcinogenic and teratogenic effects of aquatic pollutants (Pliss et al., 1982, Mizell and Romig, 1997, Oberemm, 2000). In this respect, fish are especially noted for experimentally-induced neoplastic responses in liver reviewed by Hinton and Couch, 1998). Besides hepatocarcinomas, such as in tilapia (Ding et al., 1989), there were reported cases of  
20 olfactory neuroepithelioma in domestic carp (Ishikawa et al., 1978) and abdominal sarcoma in koi carp (Lewbart et al., 1998), plasmacytoid leukemia of a retroviral origin in chinook salmon (Kent et al., 1997) and lymphosarcoma of unknown origin in brook trout (Earnest-Koons et al., 1997). Quite separately stands a very elaborate study of malignant melanomas in platyfish caused by a dominant oncogene *ONC-*

Xmark which is a tyrosine kinase receptor gene (Schartl et al., 1985). A model for melanogenesis and tumor formation in fish in particular, was proposed (Morizot et al., 1998). Accordingly, the literature supports the study cancerogenesis in small fish model systems, such as ZF. Recently, ZF was proposed as a model for human blood disorders such as congenital sideroblastic anemia and hepatoerythropoietic porphyria (Brownlie et al., 1998), (Wang et al., 1998). The data presented herein indicates that the ZF may serve as an experimental model of leukemia development as well.

Figure 3A-3B demonstrate this phenotypic change. Figure 3A shows a normally pigmented larva, while Figure 3B shows a larva with a/oligochromemia. Primitive circulating erythrocytes extracted from the fry with a/oligichromemia were indistinguishable in size and shape from the normal ones, and the loss of pigmentation was the only alteration of blood cell phenotype observed in these fish. Fry with oligochromemia were found viable and were raised and further analyzed separately from the others.

### Example 6

#### Screening potential candidate drugs for therapeutically effective agent

The animal model of the invention can be used to screen candidate compounds for therapeutic utility in the treatment and/or prevention of lymphatopoietic and hemapoietic disorders, including leukemias. As discussed in the examples above, the insertion of the transgene IK4 into zebrafish embryos markedly altered the normal cellular differentiation pattern and is correlated with the

development of hematopoietic disorders, including multilineage hematopoiesis, erythroid hyperplasia, and the like.

Administration of a putative therapeutic agent to the animal model provides an efficient, cost-effective, and reliable method for screening agents for candidates likely to improve outcome and symptoms of hematopoietic disorders, including leukemias.

### Example 7

#### Screening Potential Carcinogens

The animal model for the invention can be used to screen suspected carcinogenic agents, or agents suspected of inducing lymphatopoietic or hepatopoietic disorders. For example, ZF embryos expressing the Ik-2 transgenes produce and develop normal B- and T- lineage cells. Agents that might disrupt normal Ik-2 or other regulating controls for normal hematopoietic cell development can be efficiently and rapidly screened by administering the suspected agent to the embryo, as demonstrated by administration of Ik-4 in the Examples above.

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**WE CLAIM:**

1. An animal model for lymphocyte development and leukemia, comprising a transgenic zebrafish expressing a heterologous Ikaros protein.
2. The animal model of claim 1, wherein the Ikaros protein is a non-DNA binding form of Ikaros.
3. The animal model of claim 2, wherein the Ikaros protein lacks at least one N-terminal zinc finger domain as compared with DNA-binding forms of Ikaros.
4. The animal model of claim 3, wherein the Ikaros protein is one or more of Ik-4, Ik-5, Ik-6, Ik-7, and Ik-8, .
5. The animal model of claim 3, wherein the Ikaros protein is a deletion mutant lacking the following Ikaros amino acid sequence: KSSMPQKFLG [SEQ ID NO: 5].
6. The animal model of claim 3, wherein the Ikaros protein contains an insertion of the following amino acid sequence: VTVGADDFRDFHAIIPKSFSR [SEQ ID NO: 6].
7. An assay method for screening potential therapeutic agents useful for treating or preventing hematopoietic disorders, the assay comprising contacting a

transgenic zebrafish embryo with a potential therapeutic agent, the transgenic zebrafish embryo expressing a non-DNA binding form of Ikaros, and correlating improved lymphohematopoiesis versus a non-treated control with an effective therapeutic agent.

8. The assay of claim 7, wherein the non-DNA binding form of Ikaros protein lacks at least one N-terminal zinc finger domain as compared with DNA-binding forms of Ikaros.
9. The assay of claim 7, wherein the Ikaros protein is one or more of Ik-4, Ik-5, Ik-6, Ik-7, and Ik-8..
10. The assay of claim 7, wherein the Ikaros protein is a deletion mutant lacking the following Ikaros amino acid sequence: KSSMPQKFLG [SEQ ID NO: 5]
11. The assay of claim 7, wherein the Ikaros protein contains an insertion of the following amino acid sequence: VTVGADDFRDFHAIIPKSFSR [SEQ ID NO: 6].
12. The assay of claim 7, wherein said improved lymphohematopoiesis is analyzed in the developing zebrafish embryo.
13. The assay of claim 12, wherein said improved lymphohematopoiesis comprises improved oligochromemia in the circulating blood cells of the animal model.

14. The assay of claim 7, wherein said improved lymphohematopoiesis is analyzed in adult zebrafish.
15. The assay of claim 7, wherein said improved lymphohematopoiesis comprises improved cellularity and cellular composition of adult zebrafish kidney imprints.
16. The assay of claim 7, wherein said improved lymphohematopoiesis comprises one or more of lessened multilineage hematopoiesis, reduced erythroid hyperplasia, and reduced numbers of lymphoblasts as compared with control animals.

FIG. 1A1

A1



FIG. 1A2

A2

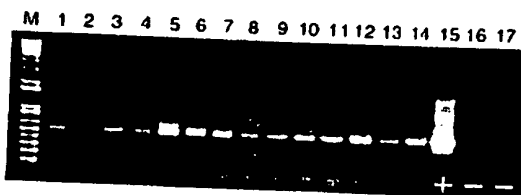


FIG. 1A3

A3

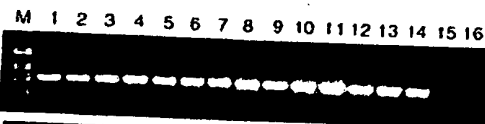


FIG. 1B1

B1



FIG. 1B2

B2

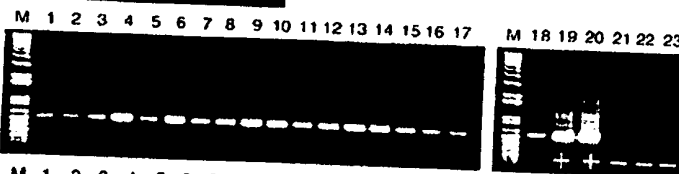


FIG. 1B3

B3

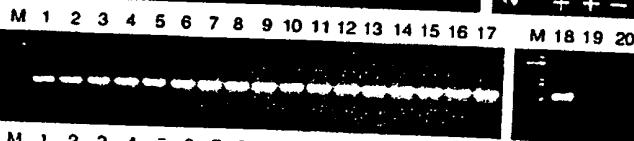
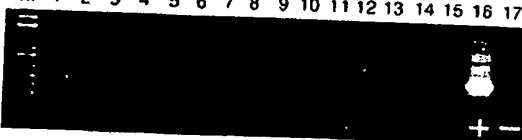


FIG. 1B4

B4



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FIG. 1C1

C1



FIG. 1C2

C2

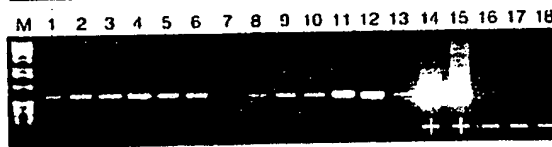


FIG. 1C3

C3

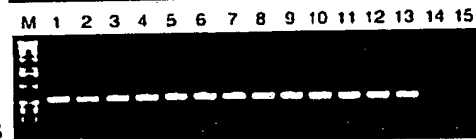


FIG. 1D1

D1

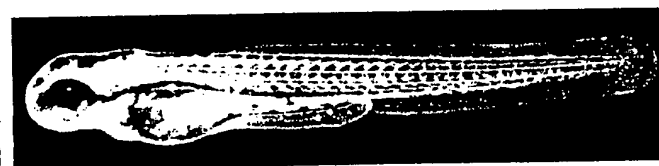


FIG. 1D2

D2

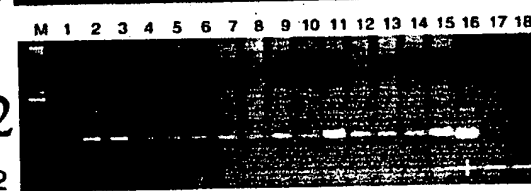
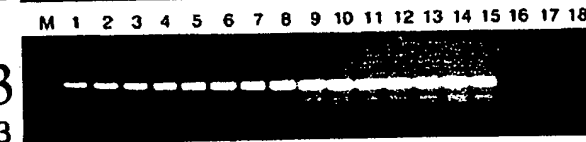


FIG. 1D3

D3



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FIG. 2A

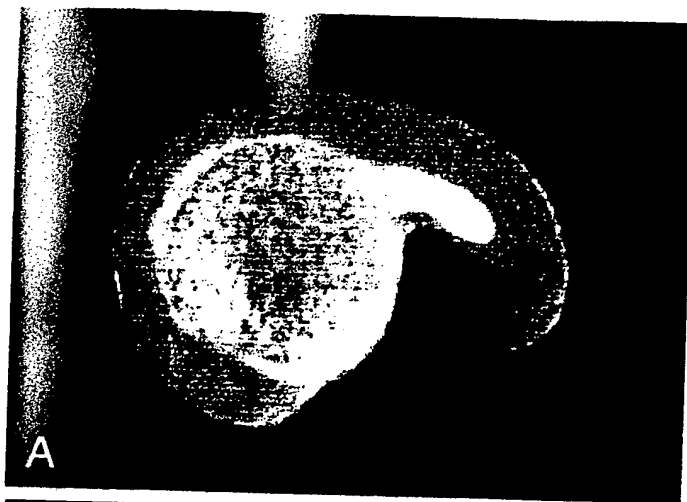


FIG. 2B

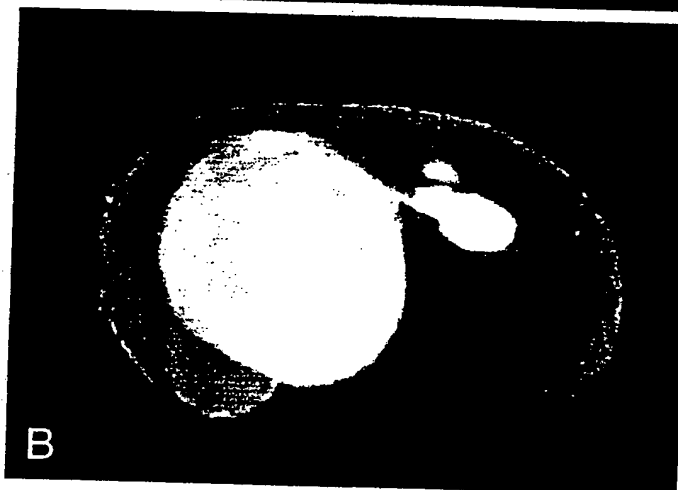


FIG. 2C





FIG. 2D

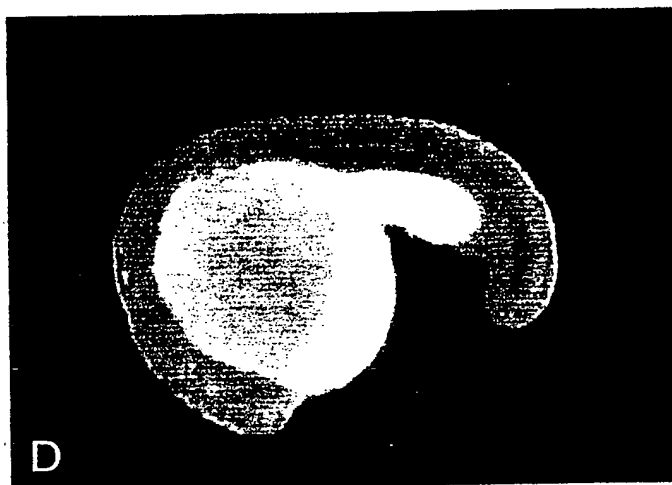


FIG. 2E

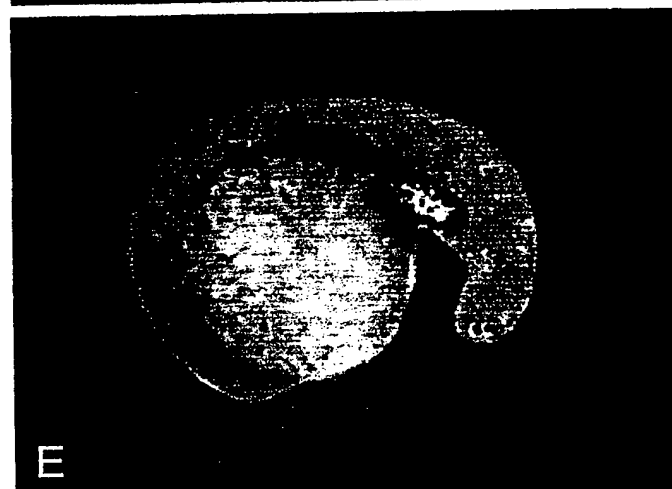
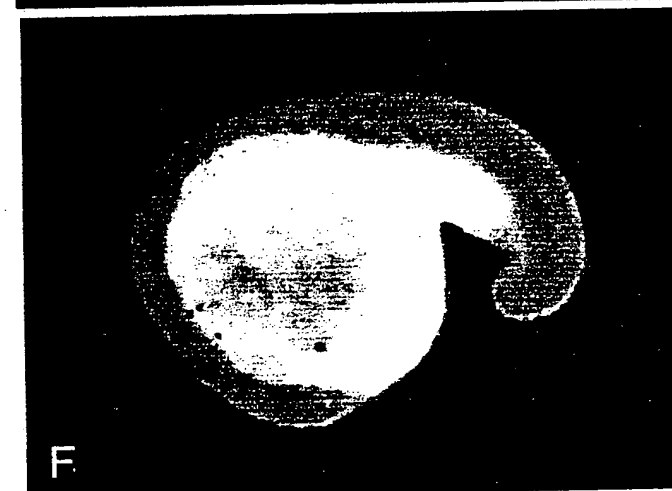


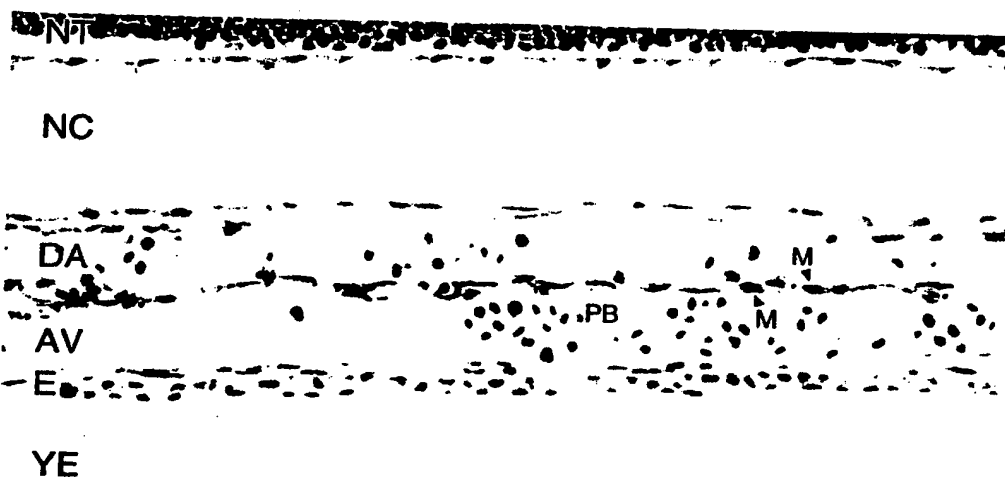
FIG. 2F



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FIG. 3A



A

FIG. 3B



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FIG. 3C



FIG. 3D

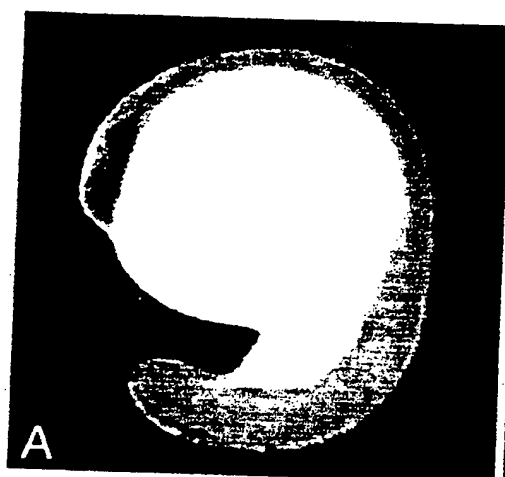


FIG. 3E

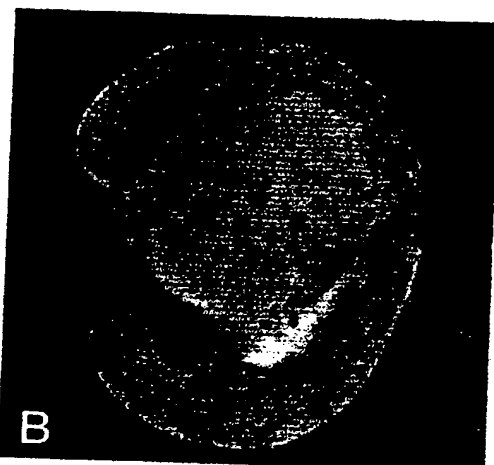


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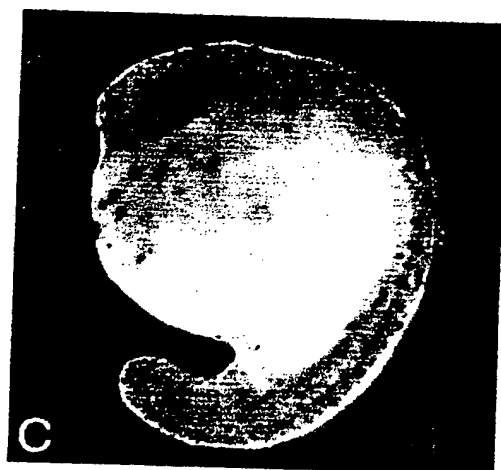
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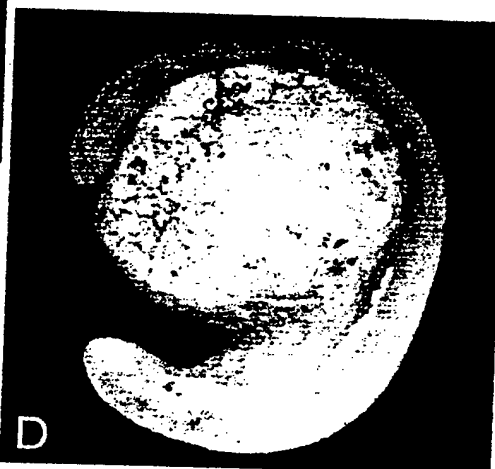
A  
FIG. 4A



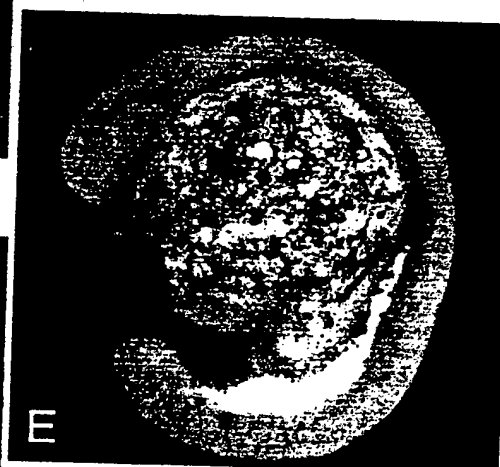
B  
FIG. 4B



C  
FIG. 4C



D  
FIG. 4D

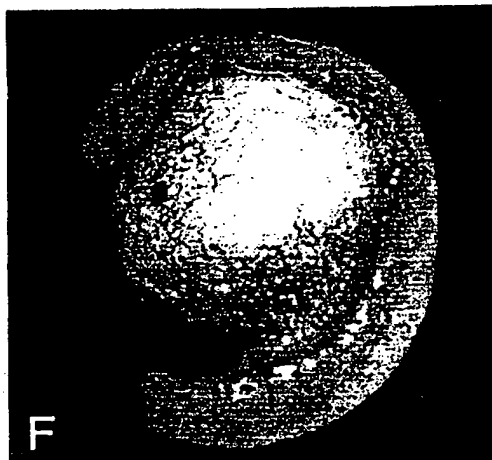


E  
FIG. 4E

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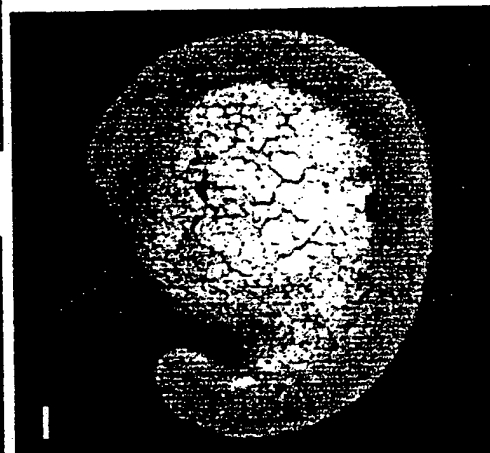
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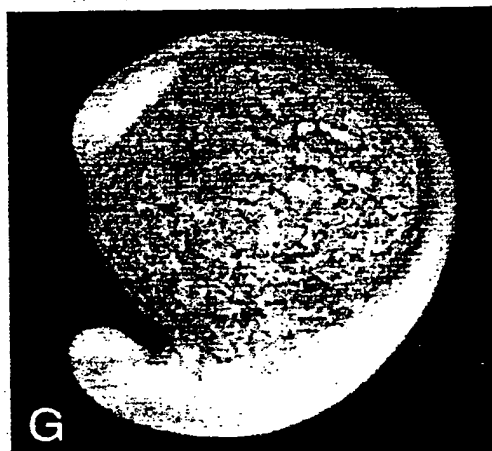
F

FIG. 4F



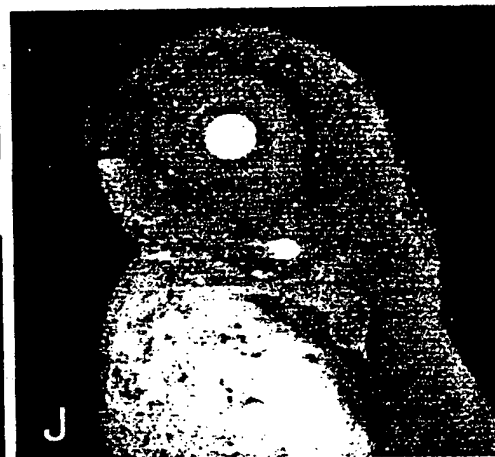
I

FIG. 4I



G

FIG. 4G



J

FIG. 4J

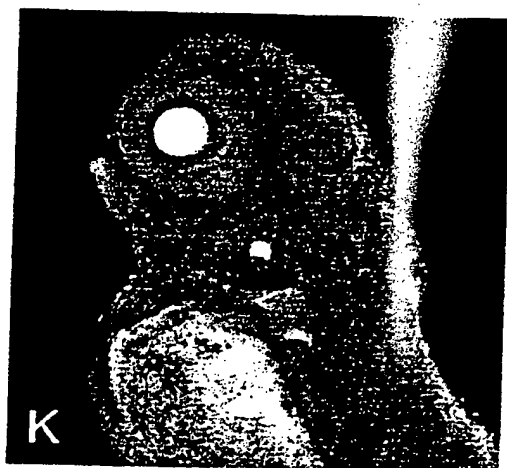


H

FIG. 4H

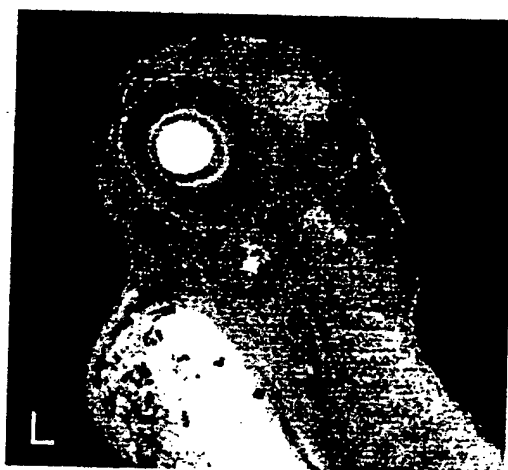
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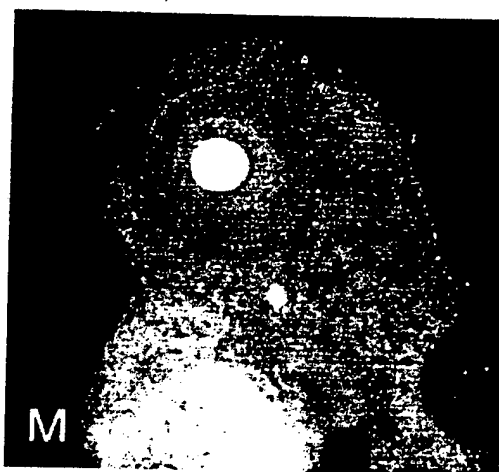
K

FIG. 4K



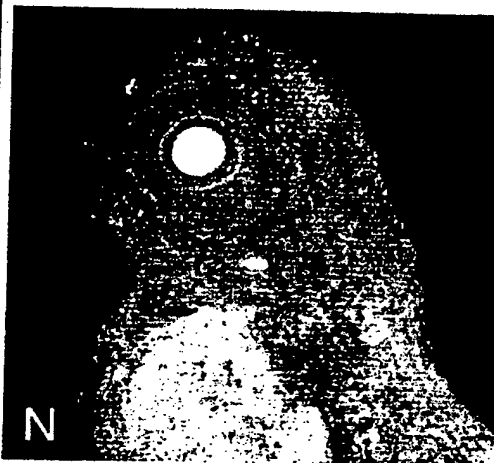
L

FIG. 4L



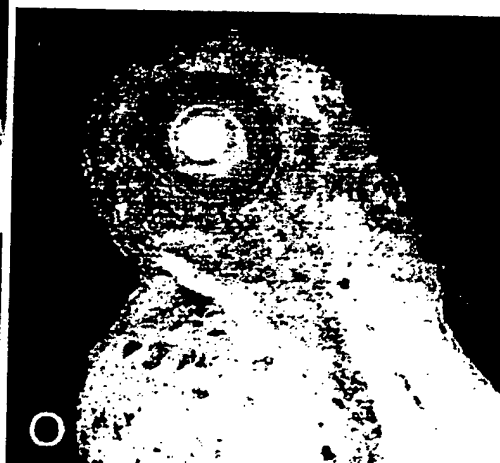
M

FIG. 4M



N

FIG. 4N



O

FIG. 4O

FIG. 5A

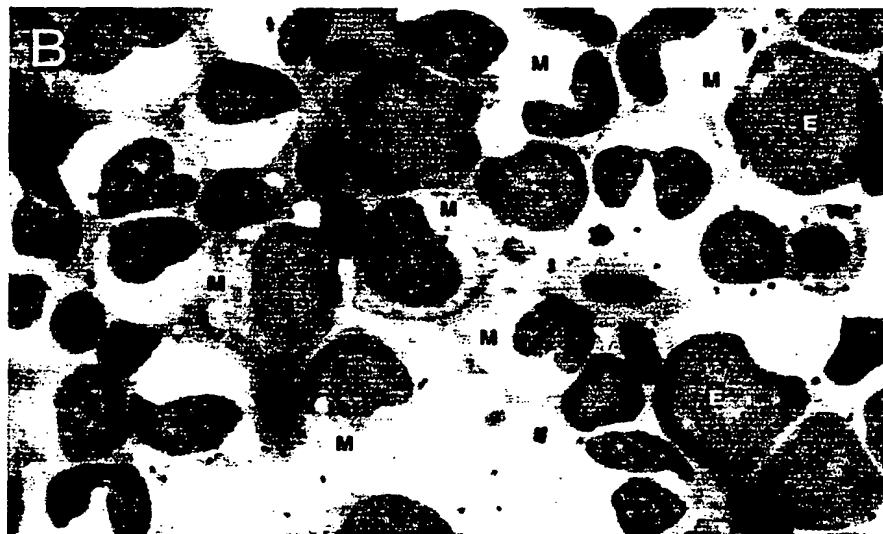
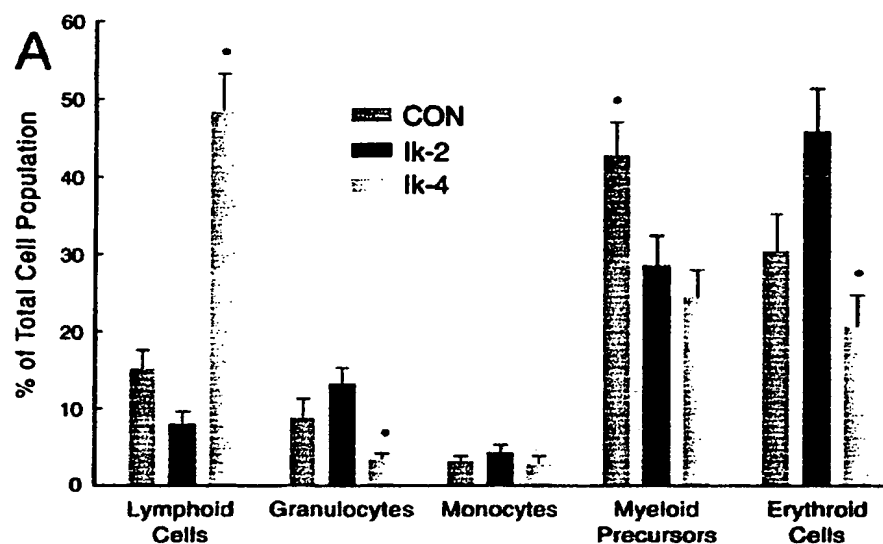


FIG. 5B

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FIG. 5C

C



FIG. 5D

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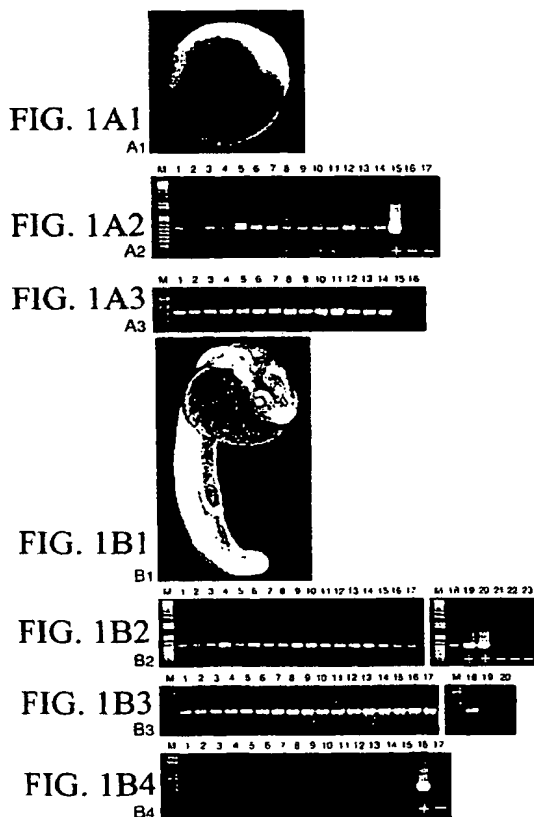
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[Continued on next page]

(54) Title: **TRANSGENIC ZEBRA FISH EMBRYO MODEL FOR HEMATOPOIESIS AND LYMPHOPROLIFERATIVE DISORDERS**

(57) Abstract: A transgenic zebrafish animal model for the study of haemopoietic cell differentiation, control, and screening of therapeutic agents.



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IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF,  
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## INTERNATIONAL SEARCH REPORT

National Application No

PC1/US 00/32757

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C07K14/47 A01K67/027

According to International Patent Classification (IPC) or to both national classification and IPC

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Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07K C12N

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Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, EPO-Internal, WPI Data, PAJ

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BENYUMOV ALEXEY O ET AL: "Forced expression of the leukemogenic human Ikaros isoform Ik4 in transgenic zebra fish embryo during primitive hematopoiesis causes a lymphoproliferative disorder in adult fish." BLOOD, vol. 94, no. 10 SUPPL. 1 PART 1, 15 November 1999 (1999-11-15), page 467a XP000993361 Forty-first Annual Meeting of the American Society of Hematology; New Orleans, Louisiana, USA; December 3-7, 1999 ISSN: 0006-4971	1-16
Y	abstract --- -/--	5-16



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

## \* Special categories of cited documents:

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Date of the actual completion of the international search

9 May 2001

Date of mailing of the international search report

22/05/2001

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# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 00/32757

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>SUN LEI ET AL: "Expression of dominant-negative and mutant isoforms of the antileukemic transcription factor Ikaros in infant acute lymphoblastic leukemia."</p> <p>PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES, vol. 96, no. 2, 19 January 1999 (1999-01-19), pages 680-685, XP002166853</p> <p>Jan. 19, 1999</p> <p>ISSN: 0027-8424</p> <p>figure 2</p>	5,10
Y	<p>L SUN ET AL: "Gene mutations and expression of dominant-negative IKAROS isoforms in T-cell acute lymphoblastic leukemia"</p> <p>BLOOD, US, W.B. SAUNDERS, PHILADELPHIA, VA, vol. 92, 15 November 1998 (1998-11-15), page 152A XP000864668</p> <p>ISSN: 0006-4971</p> <p>abstract</p>	6,11
Y	<p>WO 96 04372 A (GEN HOSPITAL CORP)</p> <p>15 February 1996 (1996-02-15)</p> <p>claims 16-19</p>	7-16

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 00/32757

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		AU 712016 B	28-10-1999
		AU 3200195 A	04-03-1996
		CA 2196190 A	15-02-1996
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